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[RAPID COMMUNICATION]

Initiation of the Earliest Nuclear Event in Fertilization of *Paramecium* by the Microinjection of Calcium Buffer

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ABSTRACT—Microinjection of Ca^{++}/EGTA buffer into the cells of *Paramecium caudatum* induces the early micronuclear migration (EMM). The induction of EMM was observed when mating reactive cells were injected with 3.4×10^{-6} M or 1.5×10^{-5} M Ca^{++}/EGTA buffer, but not observed in non-reactive cells. In control experiments, 2.0×10^{-8} M Ca^{++}/EGTA buffer, 5 mM $MgCl_2$, 50 mM NaCl, 50 mM and 300 mM KCl solutions were injected. No significant differences were observed between the control cells and uninjected cells. These results suggest that the increase in intracellular free calcium concentration initiates the earliest nuclear event in the process of conjugation in *Paramecium*.

INTRODUCTION

Calcium plays a central role in the onset of the early event of fertilization in metazoa [5, 15, 16, 18]. The increase in free cytosolic calcium concentration $[Ca^{++}]i$ after sperm entry in eggs have been demonstrated by the microinjection of Ca^{++}-sensitive photoprotein aequorin [20, 7], and by the measurement of $[Ca^{++}]i$ with Ca^{++}-sensitive microelectrodes [2].

In *Paramecium*, the earliest nuclear event, EMM (early micronuclear migration), takes place at about 10–15 min after mating reaction [10]. To examine whether $[Ca^{++}]i$ is involved in the initiation of conjugation, the artificial induction of EMM has been done by the cold treatment and by the treatment with calcium ionophore A23187 [3, 4]. Because the cold treatment of cells of paramecium in an ice bath increases in intracellular calcium concentration [1], Cronkite proposed the calcium hypothesis that the increase in $[Ca^{++}]i$ or the decrease from the increased level to normal may trigger the EMM. However, there is no direct evidence to support that $[Ca^{++}]i$ is associated with the induction of EMM. To clarify the calcium hypothesis, a series of Ca^{++}/EGTA buffers was injected into the cells of *P. caudatum* to see whether the EMM is induced.

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MATERIALS AND METHODS

Stocks and methods

Stocks of *Paramecium caudatum*, syngen 3, used were 27aG3 (mating type O) and C103 (mating type E). The culture medium was 1.25% (w/v) fresh lettuce juice diluted with K-DS (Dryl's solution modified by the substitution of KH_2PO_4 for NaH_2PO_4), pH 7.0 [6], and inoculated with *Klebsiella pneumoniae* one day before use [13]. Several hundreds of cells were inoculated into 2 ml culture medium and then fresh medium of 4 ml, 10 ml, and 10 ml was added on every successive days. Cultures were kept at 25°C. All experiments were done at room temperature (23°C). The mating reactivity of cells reached a maximum at one or two days after the final feeding.

Microinjection and EMM Assay

Microinjection was performed after the method of Koizumi [17] modified by Haga *et al* [12]. Approximately 40 pl, which is about 10% of total cell volume, was injected into the cells of C103.

The injected cells were kept in cell-free culture medium for 30 min. The cell-free culture medium used in this experiment was prepared as follows: the culture medium of C103 at stationary first day was filtered with a 0.22 μm millipore filter to remove cells. The EMM was checked under a microscope after staining with acetoorcein solution containing about 0.6% (w/v) orcein in 40% acetic acid.

Preparation of ion solutions

Following Tsiens' method, a series of Ca^{++}/EGTA buffers were prepared by combination of 10 mM EGTA and 10 mM $CaCO_3$ solution with different proportion in 10 mM HEPES, titrated with 1 N KOH to pH 7.00 at 20°C, assuming an apparent dissociation constant for the Ca^{++}/EGTA complex of 380 nM at pH 7.00 in 100 mM KCl at 20°C [11]. For control experiments, 5 mM $MgCl_2$, 50 mM NaCl, 50 mM and 300 mM KCl were prepared in 5 mM HEPES, pH 7.00.

Statistical analysis

The statistical significance of differences in mean values between control and treatments were determined by Chi square contingency tables [8]. Differences were considered to be significant for probabilities of less than 5.0%.

RESULTS AND DISCUSSION

Injection of Ca^{++}/EGTA buffers

Before the microinjection of Ca^{++}/EGTA buffers, the mating reactivity of recipient cells was tested by mixing with the mating reactive cells of the complementary mating type. The percentage of spontaneous micronuclear migration in uninjected cells was also checked by staining. Among several stocks C103 was chosen for a recipient because this strain is very stable and low in the percentage of spontaneous micronuclear migration. The clones showing strong mating reactivity and with less than 5% of micronuclear migration were used as recipients. As shown in Table 1, when the sexualy mature cells of C103 in late log-phase, so that they were non-reactive, were injected with the Ca^{++}/EGTA buffers, no significant increase in the percentage of EMM was observed. On the other hand, the microinjection of 3.4×10^{-6} M or 1.5×10^{-5} M Ca^{++}/EGTA buffer into the mating reactive cells of C103 at

TABLE 1. The relationship between $[Ca^{++}]$ injected and the occurence of EMM

Recipients	$[Ca^{2+}]$(M)	No. of exp	No. of cells	No. of EMM	% of EMM	Average % of EMM
Mating reactive	2.0×10^{-8}	1	15	1	6.7	6.1 (0.7)*
		2	15	1	6.7	
		3	19	1	5.2	
	3.4×10^{-6}	1	15	4	26.6	23.8 (2.1)*
		2	14	3	21.4	
		3	17	4	23.3	
	1.5×10^{-5}	1	15	3	20.0	22.4 (3.0)*
		2	15	4	26.7	
		3	19	4	21.0	
Uninjected		1	100	5	5.0	4.7 (0.5)*
		2	100	4	4.0	
		3	100	5	5.0	
Non-reactive	2.1×10^{-8}		20	1	5.0	
	3.4×10^{-6}		15	0	0.0	
	1.5×10^{-5}		18	0	0.0	

*, Figure in parentheses is standard deviation.

TABLE 2. Effects of injection of different cations on EMM

Cation	Con. (M)	No. of Exp.	No. of Injected cells	No. of EMM	% of EMM	Average % EMM
		1	12	1	8.3	
Mg^{2+}	5.0×10^{-3}	2	10	0	0.0	5.6 (3.7)*
		3	14	1	7.1	
		1	12	0	0.0	
Na^{+}	5.0×10^{-2}	2	8	1	12.5	6.3 (5.2)*
		3	12	1	8.3	
		1	20	1	5.0	
K^{+}	5.0×10^{-2}	2	17	1	5.9	6.5 (2.9)*
		3	9	1	11.1	
		1	12	0	0.0	
K^{+}	3.0×10^{-1}	2	15	0	0.0	0.0 (0.0)*
		3	14	0	0.0	
		1	60	3	5.0	
Uninjected cells		2	100	4	4.0	4.5 (0.4)*
		3	65	3	4.6	

*, Figure in parenthesis is standard deviation.

stationary first day induced the EMM with the percentage of 23.8% and 22.4%, respectively.

It is known that the $[Ca^{++}]i$ in *P. caudatum* is approximately 10^{-8} M–10^{-7} M [19]. Therefore, 2.0×10^{-8} M Ca^{++}/EGTA buffer was injected as the control. As shown in Table 1, statistical analysis showed a significant difference in the percentage of EMM between the cells injected with either 3.4×10^{-6} M or 1.5×10^{-5} M Ca^{++}/EGTA buffer and those injected with 2.0×10^{-8} M Ca^{++}/EGTA buffer ($P<0.05$). However, there was no significant difference between the cells injected with 2.0×10^{-8} M Ca^{++}/EGTA buffer and the uninjected cells.

Injection of Mg^{++}, Na^{+} and K^{+} solutions

To clarify whether the induction of EMM is Ca^{++} specific or not, 5 mM $MgCl_2$, 50 mM NaCl, 50 mM and 300 mM KCl solutions were injected. The results were shown in Table 2. No significant increase in the percentage of EMM was observed by the injection of these ions. Present studies have directly shown that the increase in $[Ca^{++}]i$ induces the EMM. However, efficiency of the EMM induction was not so high as that by the mating reaction. There may be several factors possibly related to the induction of EMM. First, it has been demonstrated that the percentage of EMM in normal conjugation was proportional to that of the pair formation [10]. This suggests that not only the strength of mating reactivity but also the ability to form mating pair might be required for the initiation of EMM. The cells of C103 used in this studies showed strong mating reactivity but the percentage of pair formation at 2–3 hours after mixing with the cells of complementary mating type, 27aG3 (mating type O), was about 35% among 457 cells tested. Second, sensitivity to $[Ca^{++}]i$ may be different at different places in the cytoplasm, so that the injected place would affect on the efficiency of EMM induction. Third, it is feasible that the mating reactivity of recipient cells may be reduced or lost during microinjection resulting in the decrease of sensitivity to the calcium injection. In fact, when the mating reactive cells of C103 were injected with about 40 pl of 1.5×10^{-5} M Ca^{++}/EGTA buffer, about 75% of cells ($n=12$) lost their mating reactivity at about 30 second after the microinjection. The percentage of mating reactive cells in uninjected C103 which were treated with the same way as the injected cells except microinjection was about 82%

(n=11). Fourth, the leakage of the injected Ca^{++}/EGTA buffer may happen.

In vegetative growth phase, the micronucleus usually exists in the concavity of the macronucleus. The electron microscopic studies have shown that there are microtubules between the micronucleus and the macronucleus [14]. Fujishima and Hiwatashi have demonstrated that colchicine treatment lowers the proportion of EMM. Based on this experiment, they suggested that their result might be due to the inhibition of microtubule elongation or dispersion [10]. However, our results described here provides an alternative interpretation. It is well known that a very low concentration of Ca^{++} (c.a. 6.0×10^{-6} M) depolymerizes microtubules [21]. If the Ca^{++}/EGTA buffer keeps $[Ca^{++}]i$ at the same concentration as indicated in the cytoplasm after microinjection, increase in free Ca^{++} could induce depolymerization of microtubules which connect the micronucleus with the macronucleus resulting in the micronuclear migration from the concavity of the macronucleus.

In the process of conjugation, premeiotic DNA synthesis in the migrated micronucleus starts at about 1.5 hr after the mating reaction [9]. If the pair formation is inhibited before the initiation of premeiotic DNA synthesis, the micronucleus goes back to the concavity of the macronucleus. To follow the fate of the micronucleus after EMM by Ca^{++}/EGTA buffer injection, the cells was stained at about 1.5, 2.5, and 3.0 hr after the microinjection of 3.4×10^{-6} M Ca^{++}/EGTA buffer. When micronuclei start premeiotic DNA synthesis in normal conjugation, they show an increase in volume, called the stage of swelling [9]. In this experiment, although 44.4% of the recipient showed EMM until about 3.0 hr after the injection (n=18), the apparent morphological change suggesting the premeiotic DNA synthesis in micronuclei was not observed. In the normal conjugation of C103, about 60% of cells showed the stage of swelling at about 2.0 hr after mating reaction (n=142). However, because the swelling of micronucleus is not a clear criterion for the detection of premeiotic DNA synthesis, further experiments should be done by using with labeled-nucleotide precursors or micronucleus-specific marker genes. It should be one of the most important subjects to know whether Ca^{++} is involved in the initiation of premeiotic DNA synthesis in the fertilization of Paramecium.

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